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Human Mpn1 promotes post-transcriptional processing and stability of U6atac

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ABSTRACT

Mpn1 is an exoribonuclease that modifies the spliceosomal small nuclear RNA (snRNA) U6 by trimming its oligouridine tail and introducing a cyclic phosphate group (>p). Mpn1 deficiency induces U6 3′ end misprocessing, accelerated U6 decay and pre-mRNA splicing defects. Mutations in the human *MPN1* gene are associated with the genodermatosis Clericuzio-type poikiloderma with neutropenia (PN). Here we present the deep sequencing of the >p-containing transcriptomes of *mpn1*Δ fission yeast and PN cells. While in yeast U6 seems to be the only substrate of Mpn1, human Mpn1 also processes U6atac snRNA. PN cells bear unstable U6atac species with aberrantly long and oligoadenylated 3′ ends. Our data corroborate the link between Mpn1 and snRNA stability suggesting that PN could derive from pre-mRNA splicing aberrations.

1. Introduction

Clericuzio-type poikiloderma with neutropenia (PN) is a rare recessive inherited genodermatosis characterized by early onset poikiloderma, non-cyclic neutropenia, pachyonychia, palmoplantar hyperkeratosis and skeletal defects [1–4]. PN patients carry homozygous or compound heterozygous mutations in the human gene *C16ORF57*, which encodes the evolutionarily conserved RNA exonuclease human Mpn1 (hMpn1, also dubbed Usb1) [5–8]. Mpn1 proteins were originally assigned to the 2H phosphodiesterase superfamily and indeed they contain two typical tetrapeptide motifs H-X-T/S-X, where X is a hydrophobic residue [4,9]. Yet, crystal structure and in vitro biochemical data have shown that Mpn1 proteins are 3′-to-5′ single stranded RNA exonucleases with a metal-independent catalytic activity generating a 2′,3′ cyclic phosphate (>p) group at the 3′ end of the reaction product [5,7]. hMpn1 enzymatic activity is completely abolished in mutant recombinant proteins carrying histidine-to-alanine substitutions within both tetrapeptide motifs and the majority of the mutations so far identified in PN patients lead to expression of truncated proteins lacking at least the most C-terminal tetrapeptide motif [4–8].

The only cellular target of Mpn1 identified so far is the spliceosome core component U6 snRNA. U6 is produced by RNA polymerase III (RNPIII)-mediated transcription and primary U6 transcripts comprise an oligouridine (oligo(U)) tail at their 3′ ends containing 4 template Us [10,11]. Upon transcription, U6 is thought to be extended by oligouridylation executed by the enzyme TUTase [12,13] and successively Mpn1 trims the elongated oligo(U) tail to 5 Us blocked by a >p group [5–7,14]. The terminal >p is stable in humans and in *Schizosaccharomyces pombe* while it seems to be immediately cleaved to generate a 2′ or 3′ −p group in *Saccharomyces cerevisiae* [5–7]. Indeed, in normal cells, the majority of cellular U6 terminates with 5 Us blocked by either −p or >p groups [10]. On the contrary, in all Mpn1-deficient model systems tested so far, including cells established from PN patients, U6 carries longer oligo(U) tails lacking phosphate groups and terminating with *cis* 2′,3′-diol end (—OH) groups instead. Moreover, in Mpn1 deficient cells, U6 molecules are unstable, indicating that Mpn1 counteracts U6 degradation through cellular pathways whose molecular details still need to be elucidated [5–7]. It has also been shown that a minor fraction of cellular U6 carries oligo(U) tails mono- or oligoadenylated at their 3′ ends in normal human cells, while in PN cells the fraction of adenylated U6 is dramatically increased [5]. Adenylation was suggested to be performed by the non-canonical poly(A) polymerase Trf4 [5].

Despite having similar effects on U6 post-transcriptional modifications, Mpn1 inactivation has different consequences in different organisms. In budding yeast, *MPN1* deletion leads to cell

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death. On the contrary, fission yeast strains deleted for *mpn1+* are viable, although they grow slower than wt cells in the cold. Both yeast loss of function models show global defects in pre-mRNA splicing and these defects, as well as the proliferation defects, can be reverted by ectopic over-expression of U6, confirming that U6 becomes limiting upon Mpn1 deficiency [6,7]. Intriguingly, human lymphoblastoid cells from PN patients do not display any obvious defects in canonical pre-mRNA splicing, suggesting that the impact of U6 misprocessing varies among different organisms or, possibly, cell types [5,7]. Indeed, morpholino-mediated Mpn1 depletion in zebrafish larvae induced aberrant splicing of a subset of pre-mRNAs encoding polypeptides required for neutrophil differentiation and development [15]. Injection of spliced mRNA in Mpn1 depleted animals rescued neutrophil developmental defects, indicating that splicing defects of a small subset of transcripts is responsible for tissue-specific abnormalities [15]. Besides U6, no other cellular substrate of Mpn1 has been identified so far, raising the question of whether all defects associated to Mpn1 deficiency arise from inappropriate U6 processing.

Here we present a next generation sequencing-based analysis of the >p-containing transcriptome of fission yeast and human cells lacking Mpn1 activity. While U6 appears to be the major if not the only substrate for Mpn1 in yeast, human Mpn1 also processes the snRNA U6atac, which is the U6-like snRNA found within the minor spliceosome [17]. Similar to U6, U6atac carries aberrantly elongated and often mono or oligoadenylated oligo(U) tails lacking terminal phosphate groups and its stability is diminished in PN cells. Our data further consolidate the proposed link between PN and improper pre-mRNA splicing.

2. Results

2.1. Deep sequencing of the >p-containing transcriptome identifies novel putative hMpn1 substrates

To isolate RNA substrates of Mpn1, we generated cDNA libraries from 3′ end >p-containing RNA species by ligating total RNA to an oligonucleotide adaptor using *Thaliana* tRNA ligase, which specifically ligates terminal >p to —OH groups [18]. For fission yeast samples, we used RNA prepared from wt and *mpn1*Δ strains [7]. For human samples, we used RNA prepared from telomerase immortalized foreskin fibroblasts established from a PN patient (PNFF cells) stably infected with retroviruses expressing hMpn1 (PNFF-hMPN1) or, as a control, with empty vector retroviruses (PNFF-ev) Ectopic hMpn1 is C-terminally tagged with an HA epitope and expression was validated by western blot analysis (see below). For sequence analysis, we applied an arbitrary cutoff and considered only features with a normalized mapped read count of at least 800 reads in Mpn1 proficient samples, and at least a 10-fold enrichment in mapped reads in Mpn1 proficient over deficient samples. Only U6 was identified as Mpn1 substrate in fission yeast samples, with reads being 44-fold more abundant in wt than in *mpn1*Δ samples (Fig. 1A). U6 reads clustered towards the 3′ terminus and 79% of them terminated with a stretch of 5 Us, confirming that the large majority of >p-containing U6 oligo(U) tails comprise 5 uridines (Fig. 1A and C).

As for the human samples, 12 transcripts passed our selection criteria (Supplemental Table S1). Eight transcripts corresponded to internal tracts of different mRNAs; the remaining 4 transcripts corresponded to U6, U5 and U6atac snRNAs and Vault RNA 1-1 (VTRNA1-1). U6atac is the U6-like snRNA found within the minor spliceosome, which catalyzes the removal of an atypical class of introns (U12-type introns) found in vertebrates, plants, insects, and some fungi [16,17]. VTRNA1-1 is a short non-coding RNA found in the evolutionary conserved Vault particles, which are involved in diverse cellular processes such as multidrug and radiation resistance, nuclear transport and immunity [19]. While reads clustered towards the 3′ end for U6, U6atac and VTRNA1-1, U5 reads were spread within the body of the transcript (Fig. 1B). The presence of >p groups on internal mRNA and U5 sequences suggest that >p groups could be generated on degraded or processed RNA species rather than at the very 3′ end of intact transcripts. We therefore focused on U6, U6atac and VTRNA1-1. Similarly to what was found for yeast U6, human U6 and U6atac 3′ ends mostly comprised 5 Us, while a less strict consensus was observed for VTRNA1-1, which often terminated with 2–4 Us (Fig. 1C).

2.2. U6atac is misprocessed in PN cells

To validate our next generation sequencing results, we performed new ligations of total RNA from PNFF cells using *Thaliana* tRNA ligase and, in parallel, using T4 RNA ligase, which only ligates terminal —OH groups. In PNFF-hMPN1 samples, a large fraction of U6 and approximately 50% of U6atac were ligated with *Thaliana*, while U6 and U6atac were ligated with T4 but not *Thaliana* ligase in PNFF-ev cells (Fig. 2A). VTRNA1-1 was efficiently ligated with T4 but not *Thaliana* ligase in both PNFF-hMPN1 and PNFF-ev samples, yet we observed a very faint band corresponding to *Thaliana*-ligated VTRNA1-1 specifically in PNFF-hMpn1 samples (Fig. 2A, high contrast). As expected, 5S rRNA, which terminates with —OH groups, was ligated by T4 RNA ligase and not by *Thaliana* (Fig. 2A). Thus, in Mpn1-proficient cells, the large majority of U6, approximately half of U6atac and a very minor fraction of VTRNA1-1 contain terminal 3′ >p groups. To our knowledge, this is the first report on the existence of terminal >p groups on U6atac and VTRNA1-1.

We then analyzed the electrophoretic mobility of U6, U6atac and VTRNA1-1 using total RNA prepared from PNFF-ev and PNFF-hMPN1 cells as well as from PNFF cells infected with three hMpn1 variants carrying alanine to histidine substitutions within their tetrapeptide motifs (hMPN1^{MA} carries the mutation H120A; hMPN1^{MB} carries the mutation H208A; hMPN1^{DM} carries both mutations). All ectopic hMpn1 variants were expressed at similar levels (Fig. 2B). In addition, we used RNA from two previously characterized lymphoblastoid cell lines established from a PN patient (P1) and his healthy brother (R1) [7,8]. As expected, U6 migration was retarded in PNFF-ev and P1 samples as compared to hMpn1-procifient counterparts (Fig. 2C). Similarly, U6atac ran more slowly in PNFF-ev and P1 samples, while VTRNA1-1 migration was not visibly affected (Fig. 2C). U6 and U6atac motilities were retarded also in PNFF-hMPN1^{MB} and PNFF-hMPN1^{DM} cells (Fig. 2B), confirming that H208 is essential for hMpn1 enzymatic activity. Unexpectedly, in PNFF-hMPN1^{MA} samples U6 and U6atac ran as in hMpn1 proficient samples (Fig. 2A), which is in strike contrast with published data showing that hMpn1^{MA} is catalytically inactive in vitro and the corresponding mutant in budding yeast cannot compensate for deletion of the *MPN1* gene [5,6]. We suggest that hMpn1^{MA} retains substantial residual activity when expressed in human cells. In this regard, it is interesting that no PN-associated mutation has been reported to affect only the first tetrapeptide motif [4].

2.3. hMpn1 processes U6atac- and VTRNA1-1-like substrates in vitro

We previously established in vitro exonuclease assays utilizing recombinant hMpn1 and hMpn1^{DM} purified from bacteria and RNA oligonucleotides mimicking the 3′ end of human U6 [7]. We performed similar assays using oligonucleotide substrates comprising the last 13 residues of U6, U6atac and VTRNA1-1 sequences terminating with 7 Us. We incubated oligonucleotides with recombinant hMpn1 proteins for increasing time periods and analyzed reaction